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Pharmacogenomic determination of genes associated with sensitivity or resistance of tumor cells to curcumin and curcumin derivatives

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Abstract

Curcuma longa L. has long been used as a medicinal plant in traditional Chinese medicine against abdominal disorders. Its active constituent curcumin has anti-inflammatory, chemopreventive and cytotoxic properties. In the present investigation, we have analyzed the cytotoxic activity of curcumin and four derivatives. Among these compounds, ethoxycurcumintrithiadiazolaminomethylcarbonate was the most cytotoxic one. The curcumin-type compounds were not cross-resistant to standard anticancer drugs and were not involved in ATP-binding cassette transporter-mediated multidrug resistance. A combined approach of messenger RNA-based microarray profiling, COMPARE analyses and signaling pathway analyses identified genes as determinants of sensitivity and resistance to curcumin and specific signaling routes involved in cellular response to curcumin. These genes may be useful as biomarkers to develop individualized treatment options in the future. From a nutritional point of view, it is a thriving perspective to further investigate whether *C. longa* may be used as a spice to improve cancer therapy.
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Keywords: Cancer; Curcumin; ABC transporter; Drug resistance; Pharmacogenomics

1. Introduction

Curcuma is a genus of the ginger family, Zingiberaceae. The best known species is *Curcuma longa* L. with its active constituent curcumin (diferuloylmethane) [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] [1]. *C. longa* L. is widely used as a spice and a coloring agent in several foods such as curry, mustard, and potato chips as well as in cosmetics and drugs [2]. Curcumin has long been used in traditional Chinese medicine against abdominal pain and jaundice [3]. It shows a wide range of pharmacological activities, including antioxidant and anti-inflammatory effects. Curcumin, particularly, has a chemopreventive effect against various human cancers [4], as high concentrations of curcumin are cytotoxic and induce apoptosis [5,6]. This might have therapeutic significance in cancer treatment. Clinical Phase I and II studies are currently being conducted. Classical chemotherapies are characterized by side effects and development of drug resistance. The development of multidrug resistance (MDR) is a major obstacle for many established cytostatic drugs, and several ATP-binding cassette (ABC) drug transporters mediate MDR [7–10]. It has been shown that *C. longa* L. as a spice and its isolated constituent curcumin are modulators of chemotherapy and radiotherapy of tumors. However, it is unknown yet whether tumor cells are able to develop resistance toward curcumin itself.

Hence, the aim of the present study was to evaluate factors that determine the response of tumors toward curcumin. For this reason, we have analyzed the role of ABC transporters for resistance to curcumin and four curcumin derivatives. We investigated cross-resistance of *P*-glycoprotein or *MRP1*-overexpressing multidrug-resistant tumor cells toward curcumin. As a next step, we analyzed cross-resistance of curcumin toward four curcumin derivatives and more than 1400 standard drugs of the Developmental Therapeutics Program of the National Cancer Institute (NCI, Bethesda, MA, USA). Since the response of tumor cells to cytotoxic agents is most frequently determined by multiple factors, it is not sufficient to analyze only ABC transporters. By using microarray-based messenger RNA (mRNA) expression profiling by COMPARE and hierarchical cluster analyses, we furthermore investigated which genes correlated with sensitivity or resistance of the NCI cell line panel toward curcumin.

2. Materials and methods

2.1. Cell lines

The panel of 60 human tumor cell lines of the Developmental Therapeutics Program of the NCI consisted of leukemia (CCRF-CEM, HL-60, K-562, MOLT-4, RPMI-

Abbreviations: ABC transporter, ATP-binding cassette (ABC) transporter; MDR, multidrug resistance; *MDR1*, multidrug resistance gene 1; MRP1, multidrug resistance-related protein 1; NCI, National Cancer Institute; TCM, traditional Chinese medicine.

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8226, SR), melanoma (LOX-IMVI, MALME-3M, M14, SK-MEL2, SK-MEL28, SK-MEL-5, UACC-257, UACC-62), non–small cell lung cancer (A549, EKVX, HOP-62, HOP-92, NCI-H226, NCI-H23, NCI-H322M, NCI-460, NCI-H522), colon cancer (COLO205, HCC-2998, HCT-116, HCT-15, HT29, KM12, SW-620), renal cancer (786-0, A498, ACHN, CAKI-1, RXF-393, SN12C, TK-10, UO-31), ovarian cancer (IGROV1, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, SK-OV-3) cell lines, cell lines of tumors of the central nervous system (SF-268, SF-295, SF-539, SNB-19, SNB-75, U251), prostate carcinoma (PC-2, DU-145) and breast cancer (MCF-7, NCI/ADR-Res, MDA-MB-231, Hs578T, MDA-MB-435, MDA-N, BT-549, T-47D). Their origin and processing have been previously described [11].

Multidrug-resistant tumor cell lines: Leukemic CCRF-CEM and HL-60 cells were maintained in RPMI-1640 medium (Invitrogen, Eggenstein, Germany) supplemented with 10% fetal calf serum in a humidified 5% CO_2 atmosphere at 37°C. Cells were passaged twice weekly. All experiments were performed with cells in the logarithmic growth phase. The *P*-glycoprotein/*MDR* gene 1 (*MDR1*)-overexpressing CEM/ADR5000 was maintained in 5000 ng/ml doxorubicin. The *MRP1*-expressing HL-60/AR subline was continuously treated with 100 nM daunorubicin. The establishment of the resistant sublines has been described [12,13]. Sensitive and resistant cells were kindly provided by Dr. J. Beck (Department of Pediatrics, University of Greifswald, Greifswald, Germany).

2.2. Drug response

The sulforhodamine B assay for the determination of drug sensitivity in the NCI cell lines has been reported [14]. The inhibition concentration 50% (IC_{50}) values for curcumin, four curcumin derivatives and over 1400 standard cytostatic drugs have been deposited in the database of the Developmental Therapeutics Program of the NCI (http://dtp.nci.nih.gov). The chemical structures of curcumin (**A**) and derivatives [curcumintritrithiadiazolaminoethylcarbonate (**B**), curcumintriadamantylaminoethylcarbonate (**C**), ethoxycurcumintribenzimidazolmethylcarbonate (**D**) and ethoxycurcumintrithiadiazolaminomethylcarbonate (**E**)] are shown in Fig. 1. These curcumin derivatives were selected because of their availability in the NCI database.

The *in vitro* response of sensitive and multidrug-resistant CCRF-CEM and HL-60 leukemia cell lines toward (**A**) (Sigma-Aldrich, Taufkirchen, Germany) was evaluated by means of a growth inhibition assay as described [15]. Aliquots of 5×10^4 cells/ml were seeded in 24-well plates, and drugs were added immediately at different concentrations. (A) was used in different doses to allow calculation of IC_{50} values. Cells were counted 7 days after treatment with the drugs. The resulting growth data represent the net outcome of cell proliferation and cell death.

2.3. Statistical analyses

The mRNA expression values of 48 ABC transporter genes in 60 cell lines were selected from the NCI database (http://dtp.nci.nih.gov). The mRNA expression has been determined by real-time reverse transcriptase polymerase chain reaction analyses as reported [16].

Hierarchical cluster analysis is an explorative statistical method and aims to group at first sight heterogeneous objects into clusters of homogeneous objects. Objects are classified by calculation of distances according to the closeness of between-individual distances. All objects are assembled into a cluster tree (dendrogram). The merging of objects with similar features leads to the formation of a cluster, where the length of the branch indicates the degree of relatedness. The procedure continues to aggregate clusters until there is only one. The distance of subordinate cluster to a superior cluster represents a criterion for the closeness of clusters as well as for the affiliation of single objects to clusters. Thus, objects with tightly related features appear together, while the separation in the cluster tree increases with progressive dissimilarity. Cluster analyses applying the WARD method were done by means of the WinSTAT program (Kalmia Co., Cambridge, MA, USA). Missing values are automatically omitted by the program, and the closeness of two joined objects is calculated by the number of data points they contained. In order to calculate the distances of all variables included in the analysis, the program automatically standardizes the variables by transforming the data with a mean=0 and a variance=1. To visualize the relationships between the IC_{50}

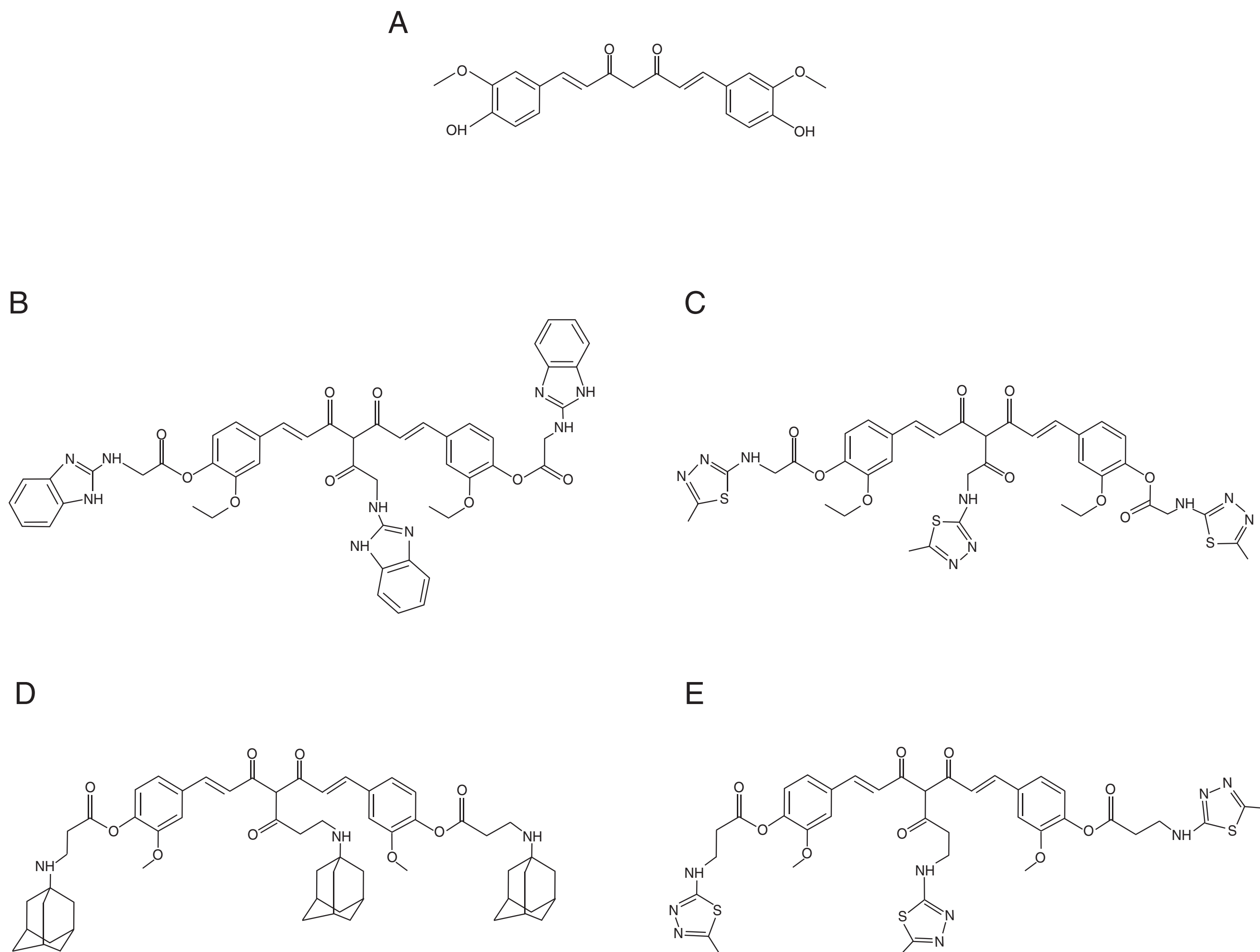

Fig. 1. Chemical structures of curcumin and derivatives: (**A**) Curcumin, (**B**) ethoxycurcumintribenzimidazolmethylcarbonate, (**C**) ethoxycurcumintrithiadiazolaminomethylcarbonate, (**D**) curcumintriadamantylaminoethylcarbonate, and (**E**) curcumintritrithiadiazolaminoethylcarbonate.

values for curcumin and mRNA expression levels by cluster analyses, cluster image maps were formed.

COMPARE analyses were performed using software implemented in the NCI Web site (http://dtp.nci.nih.gov). COMPARE analyses are rank-ordered lists of compounds. Every compound of the Standard Agent Database of the NCI is ranked for similarity of its *in vitro* cell growth pattern to the *in vitro* cell growth pattern of a selected seed or probe [17]. To derive COMPARE rankings, a scale index of similarity between the seed compound cell growth pattern and the pattern for each of the COMPARE database compounds is created. This methodology has previously been exploited to identify the presumable mode of action of investigational drugs by comparing their IC_{50} profiles of the NCI cell-line panel with those of drugs with well-established mechanisms of action [18]. We performed COMPARE analyses of the IC_{50} values for (A) and the microarray-based transcriptome-wide mRNA expression levels in 60 cell lines. First, we performed a standard COMPARE analysis, in which cell lines that were most inhibited by (**A**) (lowest IC_{50} values) were correlated with the lowest mRNA expression levels of genes. These genes may be considered as possible candidate genes, which determine cellular resistance to (**A**). Afterward, a reverse COMPARE analysis was performed: the most inhibited cell lines were correlated with the highest gene expression levels, indicating possible drug sensitivity genes.

Pearson correlation test was used to calculate significance values and rank correlation coefficients as a relative measure for the linear dependency of two variables. This test was implemented into the WinSTAT Program (Kalmia Co.). Pearson correlation test determined the correlation of rank positions of values. Ordinal or metric scaling of data is suited for the test and transformed into rank positions. There is no condition regarding normal distribution of the data set for the performance of this test. We used Pearson correlation test to correlate microarray-based mRNA expression of candidate genes with the IC_{50} values for (**A**).

The χ^2 test was applied to bivariate frequency distributions of pairs of nominal scaled variables. It was used to calculate significance values (P values) and rank correlation coefficients (R values) as a relative measure for the linear dependency of two variables. This test was implemented into the WinSTAT program (Kalmia Co.). The χ^2 test determines the difference between each observed and theoretical frequency for each possible outcome, squaring them, dividing each by the theoretical frequency and taking the sum of the results. Performing the χ^2 test necessitated to define cell lines as being sensitive or resistant to (**A**). This has been done by taking the median IC_{50} value for (**A**) as a cutoff threshold.

3. Results

3.1. Cross-resistance of curcumin and curcumin derivatives in the NCI cell-line panel

Curcumin (**A**) and four derivatives [(**B**), (**C**), (**D**) and (**E**)] have been tested over a dose range from 10^{-8} to 10^{-4} M in 60 cell lines of the NCI, and IC_{50} values have been calculated thereof. The IC_{50} values of the NCI cell line panel are shown in Fig. 2. A comparison of the IC_{50} cell lines showed that (**A**) was the least active compound and (**E**) was the most active one, while the other derivatives revealed intermediate cytotoxicity.

The IC_{50} values were subjected to Pearson correlation test. As shown in Table 1, the relationships between the five curcumin-type compounds were statistically significant, indicating that the cell lines responded similar toward these five compounds. Then, the IC_{50} values of the NCI cell line panel for these compounds were correlated with those for more than 1400 standard drugs by means of COMPARE analyses. None of the standard compounds correlated with the five curcumin compounds, with a correlation coefficient of R>0.5 and P<.05, indicating that the curcumin derivatives did not exert significant cross-resistance to standard anticancer drugs.

3.2. Correlation of IC_{50} values for curcumin-type compounds with P-glycoprotein/MDR1 and MRP1 in the NCI cell-line panel

As MDR and MDR-conferring drug transporters of the ABC transporter family are a major cause of failure to many established anticancer drugs, we addressed the question whether cellular response toward curcumin-type compounds may also be affected by ABC transporters. Therefore, we correlated the IC_{50} values of (**A**) to (**E**) with the expression of all 48 ABC transporter genes on the human genome, whose mRNA expression was reported [8]. Interestingly, significant correlations at a level of R>0.5 and P<.05 (Pearson correlation test) were not found (data not shown), indicating that the cytotoxicity of these compounds may not be hampered by ABC transporter-mediated MDR.

3.3. Lack of cross-resistance of curcumin in P-glycoprotein/MDR1- or MRP1-overexpressing cells

The role of *P*-glycoprotein/*MDR1* and *MRP1* has been exemplarily validated using cell lines that selectively overexpress these

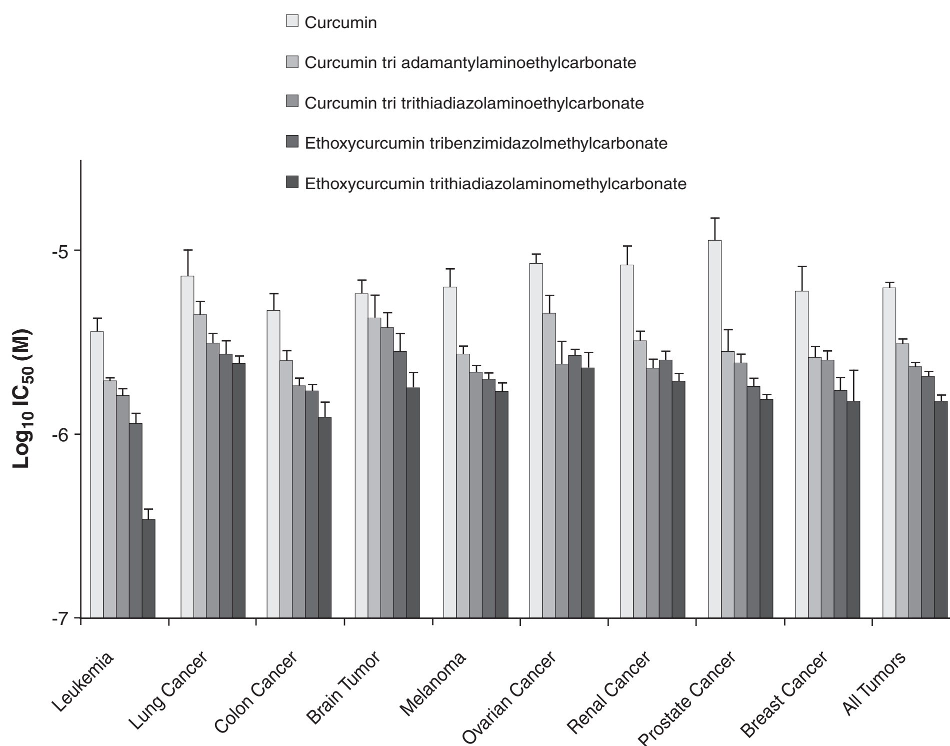

Fig. 2. IC_{50} values for the NCI cell-line panel of curcumin-type compounds calculated. Mean values and S.E.M. of IC_{50} are grouped according to the tumor origin of the cell lines.

Table 1
Cross-resistance between (**A**) and four curcumin derivatives (**B–E**) in the NCI cell-line panel

	Pearson correlation test	(**E**)	(**B**)	(**C**)	(**D**)
(**A**)	*R* value	0.51022	0.35787	0.35696	0.39714
	P value	2.14×10^{-5}	.0029	.0032	.00101
(**E**)	*R* Value		0.59755	0.62452	0.72622
	P value		2.92×10^{-7}	8.07×10^{-8}	3.77×10^{-11}
(**B**)	*R* value			0.77177	0.62567
	P value			6.64×10^{-13}	5.83×10^{-8}
(**C**)	R value				0.70598
	P value				3.01×10^{-10}

Log_{10} IC_{50} values obtained by sulforhodamine B assays have been subjected to Pearson correlation test.

genes. Based on the dose–response curves shown in Fig. 3A, *P*-glycoprotein/*MDR1*-overexpressing CEM/ADR5000 cells were not cross-resistant to (**A**) as compared with drug-sensitive parental CCRF-CEM cells. Similarly, *MRP1*-overexpressing HL60/AR cells were also not cross-resistant to (**A**) than their drug-sensitive counterparts (Fig. 3B).

3.4. COMPARE and cluster analyses of microarray-based mRNA hybridization

Next, we applied a pharmacogenomic approach to explore novel molecular determinants of sensitivity and resistance to (**A**). We mined the transcriptome-wide mRNA expression NCI database and correlated the expression data with the IC_{50} values for (**A**). This represents a hypothesis-generating bioinformatic approach, which allows the identification of novel putative molecular determinants of cellular response toward curcumin. Standard COMPARE analysis was performed to identify genes, whose expression was associated with resistance to (**A**). *Vice versa*, reverse COMPARE analysis was done to find factors associated with sensitivity of tumor cells toward (**A**). Only correlations with a correlation coefficient of $R>0.5$ (standard COMPARE) or $R<-0.5$ (reverse COMPARE) were considered (Table 2). Among the genes identified by this approach were genes from diverse functional groups such as mRNA metabolism, folate metabolism, signal transduction, DNA repair invasion, angiogenesis, apoptosis, proliferation, transporter genes and so on.

Next, the genes identified by standard and reverse COMPARE analyses were subjected to hierarchical cluster analysis. The dendrogram obtained by this procedure can be divided into three major branches (Fig. 4). The distribution of cell lines being sensitive or resistant to (**A**) was significantly different between the branches of the dendrograms. The sensitive/resistant ratio in cluster 1 was 13:15, 0:14 in cluster 2 and 16:1 in cluster 3. The distribution of cell lines among the dendrogram significantly predicted resistance to (**A**) ($P=1.14\times10-^{6}$; χ^2 test; Table 3). Interestingly, this set of genes used for cluster analysis also predicted sensitivity or resistance to (**B**) to (**E**). As shown in Table 3, significance values of $P<.05$ were obtained for all four derivatives, indicating that determinants for sensitivity or resistance were similar among all curcumin-type compounds analyzed in this study.

4. Discussion

In the present investigation, five different curcumin-type compounds have been investigated. Compared with (**A**) as lead compound, it is obvious that all derivatives are considerably larger. The esterification masks the hydrophilic phenolic hydroxyl group. This indicates that the hydrophobic features of the esterified compounds are important for binding to the pharmacophore, which might be better filled by the hydrophobic residues of the larger derivatives, (**B–E**) than by (**A**). Compared with (**B**), (**C**) and (**E**), compound (**D**) is only hydrophobic by the adamantine residue. This compound possesses, however, no possibility for additional pi-stacking as given by benzimidazole and thidiazole residues, respectively. A differentiation between (**B**), (**C**) and (**E**) is difficult without knowledge about the exact binding site. All three molecules may interact by pi-stacking. It can be assumed that (**E**) may fit best to the unknown binding site.

(**A**) has been extensively analyzed for its role in reversing MDR of tumor cells. The compound has been described to inhibit *P*-glycoprotein's efflux function both in cancer and normal cells [19–24]. In addition, (**A**) also inhibits the function of other efflux pumps of the ABC transporter family, such as Mrp1 or Bcrp (breast cancer resistance protein) [25–29] and modulates activity of ABC transporters in yeast [30]. Interestingly, (**A**) also modulates MDR of tumor cells by other mechanisms than blocking of ABC transporter function, for example, affecting the PI3K/Akt/NK-κB or FA/BRCA pathways [31,32]. (**A**) has also been reported to sensitize tumors to radiotherapy [33].

Whereas overcoming of chemoresistance and radioresistance by (**A**) is well documented in the literature, surprisingly less is known whether tumor cells can reveal resistance toward (**A**) itself and which

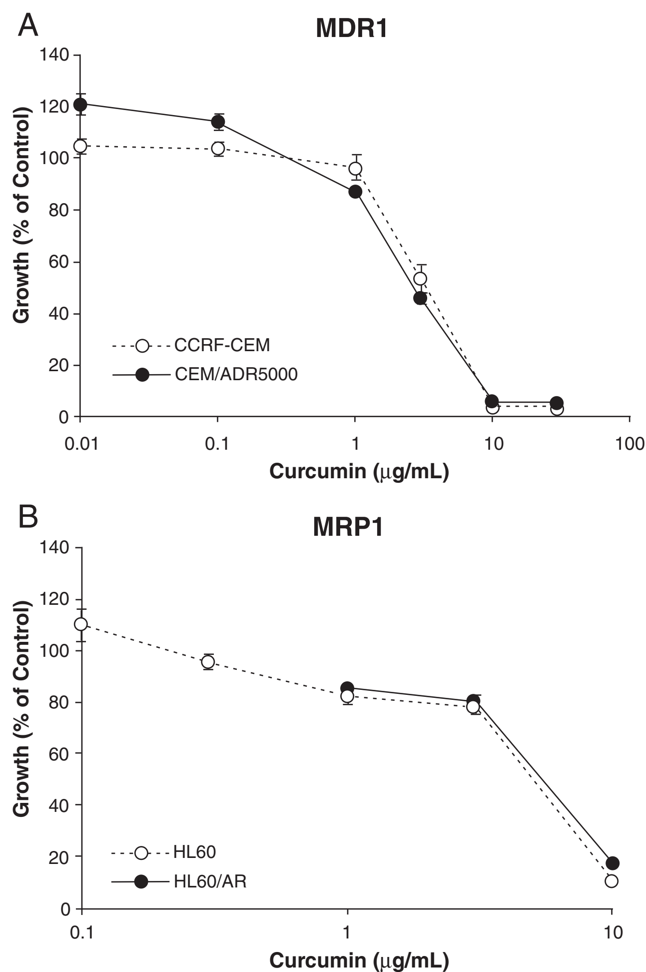

Fig. 3. Cytotoxicity of sensitive and multidrug-resistant tumor cells to curcumin. (A) Sensitive CCRF-CEM and multidrug-resistant *ABCB1* (*MDR1*)-overexpressing CEM/ADR5000 cells. (B) sensitive HL60 and multidrug-resistant *ABCC1* (*MRP1*)-overexpressing HL60/AR cells.

genes and pathways may mediate resistance to (**A**). *P*-glycoprotein/*MDR1* and Mrp1 are classical ABC transporters involved in drug resistance, while the cluster analysis of microarray data provided new determinants of resistance to (**A**). An investigation on novel factors determining sensitivity or resistance to a compound cannot neglect the analysis of well-known drug resistance factors. For this reason, we included ABC transporters as classical resistance factors to investigate their impact for (**A**). Therefore, we have correlated the mRNA expression of 48 ABC transporters of the human genome with sensitivity or resistance of the NCI tumor cell lines toward the curcumin-type compounds. Pearson correlation analyses did not reveal significant relationships ($P<.05$ and $R>0.5$). This indicates that ABC transporters may not be a major determinant of cellular resistance against (**A**). This is not only in agreement with the missing cross-resistance to 1400 standard drugs in the present investigation but also with previous investigations for one of the ABC transporters, *P*-glycoprotein, which does not confer resistance to (**A**) [34,35]. A recent report suggests that ABCA1 overexpression determines resistance to (**A**) [36]. The results of the present investigation do not support this point of view. Transport activity is determined by two different parameters: (1) functional activity and (2) expression level. The functional activity of ABC transporters is not only influenced by the number of transporter molecules (expression level). It is also affected by lipid rafts in the cell membrane. ABC transporters are surrounded by lipid rafts. If these lipid rafts contain more cholesterol, the efflux function of ABC transports is increased. This has been shown by pharmacological disturbance of *P*-glycoprotein (*ABCB1*/*MDR1*)-embedding lipid rafts by methyl-β-cyclodextrin. This compound decreases the cholesterol content of lipid rafts and leads to a decrease in pumping activity of *P*-glycoprotein [37–40]. Lipid rafts are understood as loading platforms for *P*-glycoprotein [37]. This may lead to a situation that tumor cells with stimulated lipid rafts, but low expression of *P*-glycoprotein may transport more cytotoxic molecules and are, hence, more resistant than tumor cells with *P*-glycoprotein expression but low lipid raft stimulation. This physiological condition of *P*-glycoprotein is not reflected in microarray expression experiments. Therefore, it is possible that we did not find a correlation between *ABCA1* expression and resistance to (**A**) on the basis of mRNA expression, but that Bachmeier et al. [36] found that ABCA1 transports (**A**) and confers resistance to this compound.

Since ABC transporters do not seem to play a major role for resistance to (**A**), we performed COMPARE and hierarchical cluster analyses of microarray-based mRNA expression in the NCI panel of tumor cell lines. The expression values have been reported [41] and deposited in the NCI database (http://dtp.nci.nih.gov). Indeed, it was possible to identify genes whose mRNA expression was significantly correlated with the IC_{50} values for (**A**) in the NCI cell lines. Among the specific genes that have been identified by COMPARE analyses, none have previously been associated with bioactivity of (**A**). For example, genes related to cell cycle checkpoint control and DNA damage response such as *SLC19A1* and *CHECK2*. To our knowledge, there is no available report indicating a relationship between *SLC19A1* and *CHEK2* and (**A**). However, both pathways have been reported previously to be affected by (**A**): in down-regulating genes belonging to the cell cycle and activator of transcription signaling pathways [42,43] and in promoting apoptosis in esophageal adenocarcinoma [44]. Interestingly, it has recently been shown that (**A**) indeed triggers DNA strand breaks [45].

Our pharmacogenomic approach confirms the inhibitory effect of (**A**) on biological functions, for example, cell migration and inflammation. The antimetastatic property of (**A**) might be explained by down-regulation of inflammatory cytokines *CXCL1*, *CXCL2*, cyclooxygenase-2 and also matrix metalloproteinase-2 (*MMP-2*), an anti-invasive related gene, via inhibition of NFκB [46,47]. (**A**) has been shown to inhibit *MMP-2* levels [47,48] and promote expression of *MMP-9*, a major mediator for angiogenesis and cell migration [43]. In our study, *MMP-9* belonged to the group of resistance-associated genes. The effects of (**A**) on matrix metalloproteinases were discussed in the literature. Whereas demethoxycurcumin inhibited the expression of *MMP-9* [49], (**A**) showed antiendometriosis property via down-regulation of *MMP-9* expression [50].

Hypoxia-inducible factor-1 (*HIF-1*) plays a central role in cellular responses to hypoxia, including the transcriptional activation of genes involved in angiogenesis of tumors. (**A**) inhibits hypoxia-induced angiogenesis by down-regulation of *HIF-1* [51]. In addition, (**A**) induces apoptosis and blocks migration of human medulloblastoma cells [52]. These reports can be reconciled with our data that *HIF1A* expression is associated with cellular sensitivity toward (**A**).

Folate plays an important role in DNA synthesis and methylation. Several polymorphisms in genes involved in folate uptake and biotransformation have been associated with the risk of cancer and to anticancer drug response. Expression of the *SLC19A1* gene has significantly been correlated with the sensitivity to anticancer drugs [53] and to (**A**) in the present study.

In comparison with a previously reported expression profile of apoptotic genes induced by (**A**) in human breast cancer and mammary epithelial cell lines [54], we found *DFFB* as a novel candidate gene in apoptosis signaling. This gene merits further investigation, as the influence of (**A**) on *DFFB* in apoptosis signaling is not known as yet. The *DFFB* gene encodes the DNA fragmentation factor (DFF), 40 kDa, β-polypeptide (caspase-activated DNase). The DFF described by Liu et al. [55] is a heterodimer of 40-kDa (DFFB) and 45-kDa (DFFA). DFFA is the substrate for caspase-3 that triggers DNA fragmentation during apoptosis [55]. The DFFB protein is activated during apoptosis and mediates cell-autonomous apoptotic DNA degradation (reviewed by Kawane et al. [56]).

The value of cluster analyses as the one in the present investigation is that sensitivity and resistance of tumor cells can be calculated by using a restricted set of gene expression patterns. Gene expression alone is sufficient to predict whether a tumor cell line is sensitive or resistant to a certain cytotoxic compound. Gene expression determines that resistant tumor cell lines assembled in cluster 2: 14 of 14 cell lines were resistant to the compound, and no cell line in this cluster was sensitive. This demonstrated the high accuracy to predict resistance by means of this set of gene expression. In cluster 3, re ration is inverse; that is, 16 of 17 cell lines were sensitive, and only 1 resistant cell line could not be predicted by this set of genes and was assembled in the “sensitive” cluster. The implication for the clinical situation is that with the routine diagnosis of tumors, it may be possible in the future to predict beforehand, whether a tumor would respond to chemotherapy according to the gene expression profile of the tumor.

Finally, the question arises about the effects of curcumin-type compounds to normal cells. (**B**)–(**E**) from the NCI database have not been tested on normal cells. However, the lead compound, (**A**) has been well investigated on normal cells. (**A**) reversibly inhibits normal mammary epithelial cell cycle progression by down-regulating cyclin D1 expression and by blocking its association with Cdk4/Cdk6 as well as by inhibiting phosphorylation and inactivation of retinoblastoma protein. In addition, (**A**) significantly up-regulates cell cycle inhibitory protein (p21Waf-1) in normal cells and arrests them in G(0) phase of cell cycle. Therefore, these cells escape from (**A**)-induced apoptosis at G(2) phase [57]. Pisano et al. [58] tested a curcumin derivative, the α, β-unsaturated ketone D6, which was more effective in inhibiting tumor cells growth when compared with (**A**). Normal fibroblasts proliferation was not affected by this treatment. The fact that (**A**) selectively kills tumor

Table 2
Genes determining sensitivity or resistance toward curcumin in the NCI cell line panel as identified by microarray mRNA expression profiling and COMPARE analysis

Gene	GenBank accession no.	Pearson	Name	Function
Resistance genes				
ATAD3B	AW593303	0.599	ATPase family, AAA domain containing 3B	Mitochondrial membrane protein that contributes to the stabilization of large mitochondrial DNA (mtDNA)–protein complexes called nucleoids.
PMPCB	N98771	0.595	Peptidase (mitochondrial processing) β	Member of the peptidase M16 family, which encodes a protein with a zinc-binding motif. It is located in the mitochondrial matrix and catalyzes the cleavage of leader peptides of precursor proteins newly imported into the mitochondria.
RBBP4	X74262	0.575	Retinoblastoma binding protein 4	This gene encodes a ubiquitously expressed nuclear protein that belongs to a highly conserved subfamily of WD-repeat proteins. It is present in protein complexes involved in histone acetylation and chromatin assembly. It is part of the Mi-2 complex, which has been implicated in chromatin remodeling and transcriptional repression associated with histone deacetylation. This encoded protein is also part of co-repressor complexes, which is an integral component of transcriptional silencing. It is found among several cellular proteins that bind directly to retinoblastoma protein to regulate cell proliferation. This protein also seems to be involved in transcriptional repression of E2F-responsive genes.
MMP-9	T41204	0.568	Matrix metallopeptidase 9	MMPs are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. The enzyme encoded by the MMP-9 gene degrades type IV and V collagens. The enzyme is involved in IL-8-induced mobilization of hematopoietic progenitor cells and in tumor-associated tissue remodeling.
HNRNPR	AF000364	0.56	Heterogeneous nuclear ribonucleoprotein R	This gene belongs to the subfamily of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs). The hnRNPs are RNA-binding proteins and they complex with heterogeneous nuclear RNA (hnRNA). These proteins are associated with pre-mRNAs in the nucleus and influence pre-mRNA processing and other aspects of mRNA metabolism and transport. While all hnRNPs are present in the nucleus, some seem to shuttle between nucleus and cytoplasm. The protein encoded by the *HNRNPR* gene has three repeats of quasi-RRM domains that bind to RNAs and also contains a nuclear localization motif.
ATAD3A	AL043161	0.557	ATPase family, AAA domain containing 3A	Mitochondrial membrane proteins that contribute to the stabilization of large mtDNA–protein complexes called nucleoids.
DFFB	AF064019	0.547	DNA fragmentation factor-β polypeptide	The apoptotic process is accompanied by shrinkage and fragmentation of the cells and nuclei and degradation of the chromosomal DNA into nucleosomal units. DFF is a heterodimeric protein of 40-kDa (DFFB) and 45-kDs (DFFA) subunits. DFFA is the substrate for caspase-3 and triggers DNA fragmentation during apoptosis. DFF becomes activated when DFFA is cleaved by caspase-3. The cleaved fragments of DFFA dissociate from DFFB, the active component of DFF. DFFB has been found to trigger both DNA fragmentation and chromatin condensation during apoptosis.
SFRS4	L14076	0.54	Splicing factor, arginine/serine-rich 4	Member of the arginine/serine-rich splicing factor family. The encoded protein likely functions in mRNA processing and has a probable role in alternative splice site selection during pre-mRNA splicing.
DCP1A	AA679424	0.537	DCP1 decapping enzyme homolog A	Decapping is a key step in general and regulated mRNA decay. The protein encoded by the DCP1A gene is a decapping enzyme. This protein and another decapping enzyme form a decapping complex, which interacts with the nonsense-mediated decay factor hUpf1 and may be recruited to mRNAs containing premature termination codons. This protein also participates in the TGF-β signaling pathway.
MTHFD2	X16396	0.532	Methylenetetrahydrofolate dehydrogenase 2	The *MTHFD2* gene encodes a nuclear-encoded mitochondrial bifunctional enzyme with methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase activities. The enzyme functions as a homodimer and is unique in its absolute requirement for magnesium and inorganic phosphate. Formation of the enzyme–magnesium complex allows binding of NAD.
AK2	U54645	0.526	Adenylate kinase 2	Adenylate kinases are involved in regulating the adenine nucleotide composition within a cell by catalyzing the reversible transfer of phosphate groups among adenine nucleotides. Three isozymes of adenylate kinase have been identified in vertebrates. Isozyme 2 (AK2) is localized in the mitochondrial intermembrane space and may play a role in apoptosis. It catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP. The enzyme is involved in energy metabolism and nucleotide synthesis that is essential for maintenance and cell growth. It also plays a key role in hematopoiesis.
TAF12	X84002	0.519	TAF12 RNA polymerase II	Control of transcription by RNA polymerase II involves the basal transcription machinery. TAFs are components of the transcription factor IID (TFIID) complex, PCAF histone acetylase complex and TATA box-binding protein (TBP)-free TAFII complex (TFTC). TAFs components TIIFD are essential for mediating regulation of RNA polymerase transcription. TAFs are predicted to mediate the function of distinct transcriptional activators for a variety of gene promoters and RNA polymerases. TAF12 interacts directly with TBP as well as with TAF2I.
PPP1R8	W74099	0.515	Protein phosphatase 1, regulatory subunit 8	Inhibitor subunit of the major nuclear protein phosphatase-1 (PP-1). It has RNA-binding activity but does not leave RNA and may target PP-1 to RNA-associated substrates. It may be involved in pre-mRNA splicing and binds DNA acting as transcriptional repressor. It seems to be required for cell proliferation.
CHEK2	AF086904	0.514	CHK2 checkpoint homolog	Checkpoint kinases (Chks) are serine/threonine kinases that are involved in the control of the cell cycle. Two subtypes have so far been identified, Chk1 and Chk2, which are encoded by the *CHEK1* and *CHEK2* genes, respectively. They are essential components to delay cell cycle progression in normal and damaged cells and can act at all three cell cycle checkpoints. Chks are activated by phosphorylation. ATR kinase phosphorylates Chk1 in response to single-strand DNA breaks and ATM kinase phosphorylates Chk2 in response to double-strand breaks. Chks phosphorylate Cdc25 phosphatase at Ser216, which leads to Cdc25 sequestration in the cytoplasm. Therefore, Cdc25 cannot remove the inhibitory phosphorylation on mitotic promoting factor, and entry into mitosis is prohibited. In addition, Chks have a role in the physiological stress of hypoxia/reoxygenation.

Table 2 (*continued*)

Gene	GenBank accession no.	Pearson	Name	Function
RABEPK	NM_005833	0.514	Rab9 effector protein with kelch motifs	The Rab9 effector is encoded by the *RABEPK* gene and is required for endosome to *trans*-Golgi network transport.
NFATC2IP	AA152202	0.507	Nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2 interacting protein	The protein encoded by this gene play a role in the inducible expression of cytokine genes in T cells by increasing NFAT-driven IL-4 production.
BTAF1	AF038362	0.504	BTAF1 RNA polymerase II, B-TFIID transcription factor-associated	Initiation of transcription by RNA polymerase II requires the assistance of TBP (and TBP-associated factors, or TAFs in 2 distinct complexes, TFIID and B-TFIID). The protein encoded by the *BTAF1* gene regulates transcription in association with TBP. It removes TBP from the TATA box in an ATP-dependent manner.
PNRC2	N94924	0.504	Proline-rich nuclear receptor coactivator 2	The protein encoded by the *PNRC2* gene is involved in nonsense-mediated mRNA decay (NMD) by acting as a bridge between the mRNA decapping complex and the NMD machinery. It may act by targeting the NMD machinery to the P-body and recruiting the decapping machinery to aberrant mRNAs. It is required for UPF1/RENT1 localization to the P-body and also acts as a nuclear receptor coactivator. It may play a role in controlling the energy balance between energy storage and energy expenditure.
HNRNPA2B1	M29065	0.502	Heterogeneous nuclear ribonucleoprotein A2/B1	This gene belongs to the A/B subfamily of ubiquitously expressed hnRNPs. The hnRNPs are RNA-binding proteins, and they complex with hnRNA. These proteins are associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA processing and other aspects of mRNA metabolism and transport. The protein encoded by the *HNRNPA2B1* gene has two repeats of quasi-RRM domains that bind to RNAs. It is involved with pre-mRNA processing and forms complexes (ribonucleosomes) with at least 20 other different hnRNPs and heterogeneous nuclear RNA in the nucleus.
SLC19A1	U17566	0.5	Solute carrier family 19, member 1	Transport of folate compounds into mammalian cells can occur via receptor-mediated or carrier-mediated mechanisms. A functional coordination between these 2 mechanisms mediates folate uptake. The protein encoded by the SLC19A1 gene is a transporter for the intake of folate.
Sensitivity genes				
RASAL2	W46352	−0.549	RAS protein activator like 2	This gene encodes a protein that contains the GTPase-activating protein (GAP)-related domain, a characteristic domain of GAPs. GAPs function as activators of Ras superfamily of small GTPases. The protein encoded by this gene is able to complement the defective RasGAP function in a yeast system.
ANXA2	D28364	−0.514	Annexin A2	This gene encodes a member of the annexin family. Members of this calcium-dependent phospholipid-binding protein family play a role in the regulation of cellular growth and in signal transduction pathways. This protein functions as an autocrine factor that heightens osteoclast formation and bone resorption. It is a calcium-regulated membrane-binding protein whose affinity for calcium is greatly enhanced by anionic phospholipids. It binds 2 calcium ions with high affinity and may be involved in heat–stress response.
HIF1A	W47003	−0.512	Hypoxia-inducible factor 1, α subunit	*HIF1* is a transcription factor found in mammalian cells cultured under reduced oxygen tension that plays an essential role in cellular and systemic homeostatic responses to hypoxia. *HIF1* is a heterodimer composed of an α subunit and a β subunit. The β subunit has been identified as the aryl hydrocarbon receptor nuclear translocator. The *HIF1A* gene encodes the α subunit of HIF-1. It functions as a master transcriptional regulator of the adaptive response to hypoxia. Under hypoxic conditions, it activates the transcription of over 40 genes, whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia. *HIF1A* plays an essential role in embryonic vascularization, tumor angiogenesis and pathophysiology of ischemic disease.
TGM2	M55153	−0.502	Transglutaminase 2	Transglutaminases are enzymes that catalyze the cross-linking of proteins by epsilon-gamma glutamyl lysine isopeptide bonds. While the primary structure of transglutaminases is not conserved, they all have the same amino acid sequence at their active sites, and their activity is calcium dependent. The protein encoded by the *TGM2* gene acts as a monomer, is induced by retinoic acid and appears to be involved in apoptosis. Finally, the encoded protein is the autoantigen implicated in celiac disease.

Information on gene functions was taken from the OMIM database, National Cancer Institute, USA. (http://www.ncbi.nlm.nih.gov/Omim/http://www.ncbi.nlm.nih.gov/Omim/), and from the GeneCard database of the Weizman Institute of Science, Rehovot, Israel (http://bioinfo.weizmann.ac.il/cards/index.htmlhttp://bioinfo.weizmann.ac.il/cards/index.html). IL, interleukin, TGF-β, transforming growth factor β.

cells, but not normal cells, has also recently been described by Ravindran et al. [59].

The present investigation highlights the significance of curcumin derivatives as a potent anticancer agent. Our results have to be seen in a broader context. Recently, Gupta et al. [60] discussed a general role of nutraceuticals that suggests that diet can prevent cancer. A large number of compounds from diet can modulate inflammatory pathways and thus affect the survival, proliferation, invasion, angiogenesis and metastasis of the tumor. Various cell signaling pathways are modulated by these agents. Hence, the full potential of natural products from diet should be used to developed valuable adjuncts to current cancer therapy options.

In conclusion, the present investigation indicates that (**A**) reveals profound cytotoxic activity toward cancer cells in addition to the plethora of reports on a chemopreventive role of (**A**). The cytotoxic activity was even improved by derivatives of (**A**), with (**E**) as the most active one in the present investigation. It is not only the considerable cytotoxic activity but also the missing cross-resistance to 1400 standard agents and noninvolvement in ABC transporter-mediated MDR, which makes this class of compound interesting candidates for cancer therapy. The mRNA-based microarray and COMPARE analyzes showed specific genes as determinants of sensitivity and resistance to (**A**), which may be useful as biomarkers to develop individualized treatment options in the future. From a

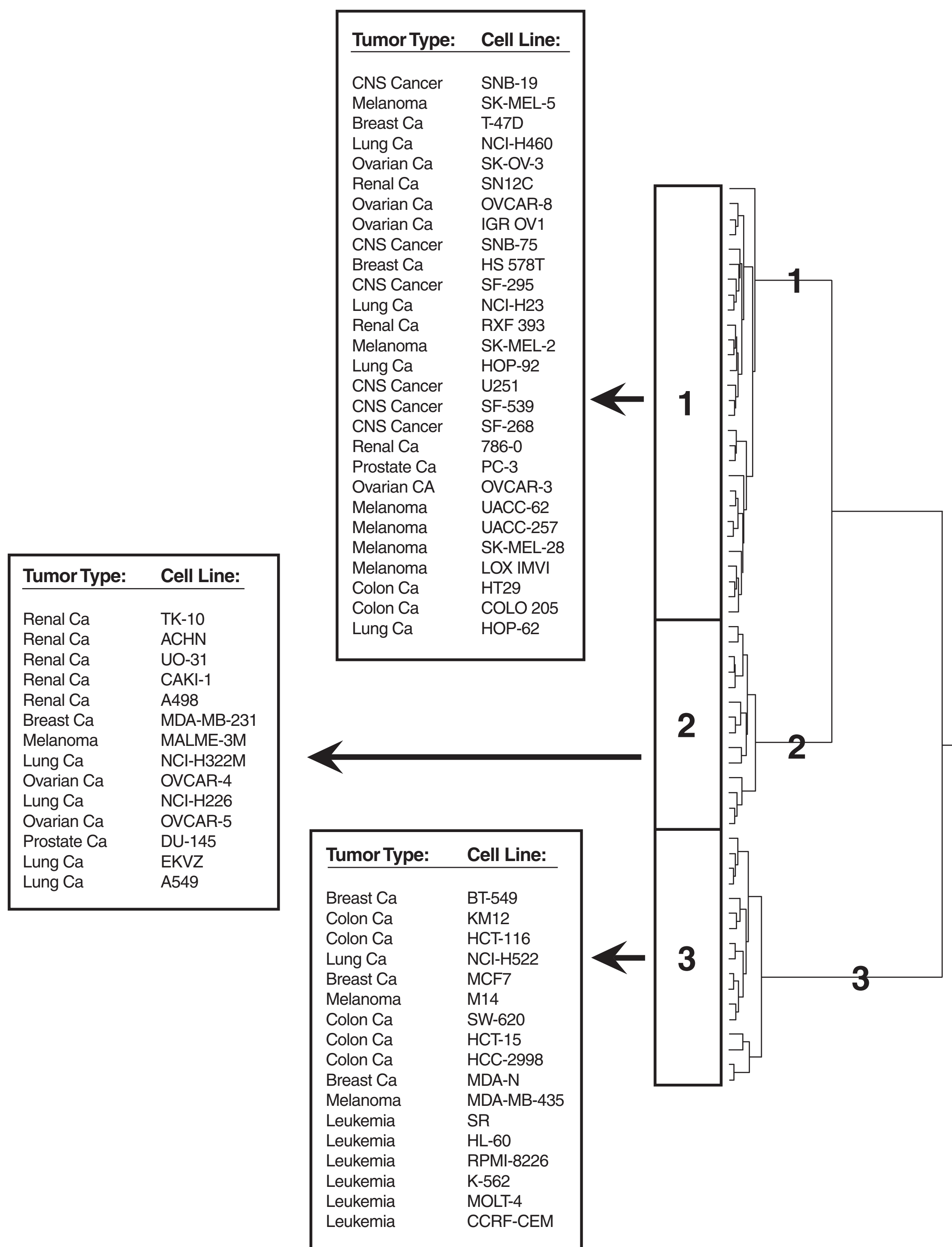

Fig. 4. Hierarchical cluster analysis of microarray-based mRNA expression of genes obtained by standard and reverse COMPARE analyses. The dendrogram shows the clustering of the NCI cell-line panel and indicates the degrees of relatedness between cell lines.

nutritional point of view, it is a thriving perspective to further investigate whether *C. longa* used as a spice might improve the response of patients to cancer therapy.

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Table 3
Separation of clusters of the NCI cell-line panel obtained by the hierarchical cluster analysis shown in Fig. 4 in comparison with drug sensitivity

Compound	Partition[a]	Cluster 1	Cluster 2	Cluster 3	χ^2 Test
(**A**) (n=59)	Sensitive (≤5.165 M)	13	0	16	P=1.14×10−[6]
	Resistant (>5.165 M)	15	14	1	
(**B**) (n=59)	Sensitive (≤5.578 M)	10	6	13	P=.009
	Resistant (>5.578 M)	19	8	3	
(**C**) (n=58)	Sensitive (<5.542 M)	9	4	11	P=.032
	Resistant (>5.542 M)	19	10	5	
(**D**) (n=59)	Sensitive (≤5.685 M)	12	5	12	P=.050
	Resistant (>5.685 M)	17	9	4	
(E) (n=59)	Sensitive (≤5.745 M)	13	4	12	P=.032
	Resistant (>5.745 M)	16	10	4	

[a] The median $\log_{10}IC_{50}$ value for each drug was used as a cutoff to separate tumor cell lines as being "sensitive" or "resistant".

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